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RESEARCH PAPER

Pp6-FEH1 encodes an enzyme for degradation of highly polymerized levan and is transcriptionally induced by defoliation in timothy (*Phleum pratense* L.)

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Abstract

The ability of grasses to regrow after defoliation by cutting or grazing is a vital factor in their survival and an important trait when they are used as forage crops. In temperate grass species accumulating fructans, defoliation induces the activity of a fructan exohydrolase (FEH) that degrades fructans to serve as a carbon source for regrowth. Here, a cDNA from timothy was cloned, named *Pp6-FEH1*, that showed similarity to wheat fructan 6-exohydrolase (6-FEH). The recombinant enzyme expressed in *Pichia pastoris* completely degraded fructans that were composed mainly of β(2,6)-linked and linear fructans (levan) with a high degree of polymerization (DP) in the crown tissues of timothy. The substrate specificity of Pp6-FEH1 differed from previously characterized enzymes with 6-FEH activity in fructan-accumulating plants: (i) Pp6-FEH1 showed 6-FEH activity against levan (mean DP 20) that was 4-fold higher than against 6-kestotriose (DP 3), indicating that Pp6-FEH1 has a preference for β(2,6)-linked fructans with high DP; (ii) Pp6-FEH1 had significant activity against β(2,1)-linked fructans, but considerably less than against β(2,6)-linked fructans; (iii) Pp6-FEH1 had weak invertase activity, and its 6-FEH activity was inhibited slightly by sucrose. In the stubble of seedlings and in young haplocorms from adult timothy plants, transcripts of *Pp6-FEH1* were significantly increased within 3 h of defoliation, followed by an increase in 6-FEH activity and in the degradation of fructans. These results suggest that *Pp6-FEH1* plays a role in the degradation of fructans and the mobilization of carbon sources for regrowth after defoliation in timothy.

Key words: Defoliation, fructan, fructan exohydrolase, levan, timothy (*Phleum pratense* L.).

Introduction

Many of the grass species used for forage, including the perennial grasses, have the ability to undergo rapid regrowth following defoliation by cutting or grazing. The meristems of forage grasses are situated near ground level, allowing the plants to regrow after defoliation. Studies on some temperate grass species have shown that defoliation-induced regrowth, following removal of photosynthetically active tissues, involves the degradation of fructans (Volenec, 1986; Gonzalez *et al.*, 1989). These fructose polymers are the main storage carbohydrates and are stored in basal tissues; regrowth rapidly stimulates fructan mobilization and alters carbon allocation. Morvan-Bertrand *et al.* (1999) reported that before the third day of regrowth, leaf growth in *Lolium perenne* mostly depends on fructan reserves, but, thereafter, it relies mainly on newly assimilated carbohydrates. The enzyme fructan exohydrolase (FEH) is responsible for the hydrolysis of fructans and releases the terminal fructose from a fructan molecule. When the degradation of fructans begins after defoliation, FEH activity increases in the stubble of temperate grasses (Mino and Maeda, 1976; Yamamoto and Mino, 1982; Prud'homme *et al.*, 1992).

Abbreviations: DPs, degrees of poymerization; DTT, dithiothreitol; FEH, fructan exohydrolase; FT, fructosyltransferase.

Partially purified FEHs from the grasses *Dactylis*, *Phleum*, *Festuca*, and *Lolium* have been described (Simpson and Bonnett, 1993; Marx *et al.*, 1997). Yamamoto and Mino (1989) reported that the increase in FEH (phleinase) activity in defoliated stem bases (pseudostems) of orchardgrass (*Dactylis glomerata* L.) derives from *de novo* synthesis of the enzyme. Over the past decade, identification and functional characterization of FEH cDNAs have been reported in several plant species. Based on analysis of the phylogenetic relationships of amino acid sequences, FEHs have been shown to be closely related to cell wall invertases, whereas fructosyltransferases (FTs), the enzymes responsible for synthesis of fructans, have similarity to vacuolar invertases (Van den Ende *et al.*, 2009). Two types of FEH activity can be distinguished depending on the linkage form of fructan on which they act: fructan 6-exohydrolase (6-FEH) activity hydrolyses β(2,6)-linked fructans; and fructan 1-exohydrolase (1-FEH) activity hydrolyses β(2,1)-linked fructans (Van den Ende *et al.*, 2004). Temperate grasses mainly accumulate β(2,6)-linked fructans (Bonnett *et al.*, 1997). A 6-FEH cDNA has been identified in wheat, which predominantly accumulates graminan type fructans composed of a mixture of fructosyl β(2,1)- and β(2,6)-linked compounds (Van Riet *et al.*, 2006). In wheat, in addition to 6-FEH, other FEH homologues have been identified: 1-FEH (1-FEH w1 and w2, Van den Ende *et al.*, 2003*a*; 1-FEH w3, Van Riet *et al.*, 2008), 6&1-FEH (6&1-FEH w1 and w2, Kawakami *et al.*, 2005), and 6-KEH (6-KEH w1 and w2, Van den Ende *et al.*, 2005). 6-FEH cDNAs have also been found in *Arabidopsis* and sugar beet, non-fructan-producing species (Van den Ende *et al.*, 2003*b*; De Coninck *et al.*, 2005), but not in temperate forage grass species. In *L. perenne,* characterization of the expression of a 1-FEHa cDNA suggested that it might play a role in the trimming of fructans during synthesis rather than in fructan mobilization (Lothier *et al.*, 2007).

In temperate grasslands that have severe winters, timothy (*Phleum pratense* L.) is used as a forage plant due to its high nutritive quality and good winter hardiness. It predominantly accumulates simple β(2,6)-linked fructans, levan with higher degrees of polymerization (DPs) (up to 90) than in other grass species (Suzuki, 1968; Cairns *et al.*, 1999). A cDNA encoding an FT involved in the synthesis of β(2,6)-linked fructans with high DPs has been identified (Tamura *et al.*, 2009). In timothy, after wintering, the lower basal internodes swell and thicken to form a structure called a haplocorm. The haplocorm stores nutrients and is useful for vegetative regrowth after cutting or grazing, because fructans comprise up to 40% of its dry matter (Sheard, 1967). Defoliation-induced fructan degradation and FEH activity in the haplocorm of timothy has been confirmed (Mino and Maeda, 1976). However, little is known about the mechanism of FEH induction, and no FEH cDNAs have been identified to date in timothy.

This study seeks to elucidate the mechanism of FEH induction after defoliation in temperate grasses by (i) cloning and functional characterization of a timothy cDNA encoding an FEH, and (ii) investigating changes in transcript levels of this *FEH* gene in the stubble of timothy seedlings and in haplocorms of adult plants after defoliation.

Materials and methods

Plant materials

Seedlings of the timothy cultivar 'Hokushu' were grown in soil in a controlled climate chamber (16 h light, 150 μmol m^{-2} s^{-1} photon flux density, 22 °C) for 6 weeks after germination. They were then used for the defoliation experiment: the seedlings were cut 5 cm above the ground 1.5 h after the beginning of the light period. Three biological replicates were sampled using 8–10 seedlings on each occasion; tissues were sampled from the cut seedlings at 2 cm above root tissues at each sampling time. Tissues from non-defoliated seedlings were used as the control.

To study haplocorms, 'Hokushu' plants were grown outdoors in 4.8-cm square pots in Sapporo, Japan from October 2009 to April 2010, and transferred to 15-cm depth pots in a greenhouse (natural light conditions) to allow additional growth. The plants were maintained at an average temperature of 17.4 °C during this period, and also after defoliation. After 6 weeks, mature plants before heading were defoliated at 5 cm above the ground at 10:00 a.m. Three biological replicates of young haplocorms originating from different genotypes were sampled at each sampling time. Haplocorms from non-defoliated plants were used as the control.

Cloning of FEH cDNA

A cDNA library was prepared previously from timothy crown tissues (see Tamura *et al.*, 2009). Partial fragments of putative FT and invertase cDNAs were obtained by PCR amplification from the cDNA library using the primers 6SFT-5F (5′-GAGATGCTG-CAGTGGCAGCG-3′) and FT-1R (5′-CCNARYGMGTAG-TAGTCGTG-3′). The primer sequences were designed using DNA sequences conserved among Poaceae FT genes. Three amplified fragments of different lengths were cloned into pGEM®-T Easy Vector (Promega) and the nucleotide sequences of the fragments were determined using the CEQ 8000 Genetic Analysis System (Beckman Coulter). A 725-bp fragment (AB436698, probe A) that had high similarity to a partial sequence of Poaceae FT genes (also used in Tamura *et al.* 2009), and a 1167-bp fragment (AB583556, probe B) that showed similarity to vacuolar invertase genes and included a putative 460-bp insertion without homology to any other genes, were used as probes. These fragments were labelled using a PCR DIG Probe Synthesis Kit (Roche) and used for cDNA library screening by plaque hybridization with a DIG Luminescent Detection Kit for Nucleic Acid (Roche). Approximately 4.0×10^4 recombinant plaques were screened using probe A and probe B. Plaques positive for probe B but either negative or slightly positive for probe A were isolated. After plaque purification, *in vivo* excision of the pBluescript SK– phagemid vector was performed in the *Escherichia coli* XLOLR strain. The nucleotide sequences of both strands of the inserts were determined. Multiple sequence alignments and phylogenetic trees were constructed by the neighbor-joining method, using the CLUSTALW program of DNASIS®Pro ver. 2.0 software (Hitachi Software Engineering).

Expression of recombinant protein in methylotrophic yeast

The isolated cDNA was expressed in the methylotrophic yeast *Pichia pastoris* (EasySelect Pichia Expression kit, Invitrogen) after cloning into the secretory expression vector pPICZαA. The DNA sequence corresponding to the putative mature protein

region predicted by SignalP 3.0 (Bendtsen *et al.*, 2004) was amplified using the primers tyft81-1F (5′-CCC-CGAATTCCTCCGCTGGCTCTCTGACCC-3′) and tyft81-1R (5′-CCCCTCTAGAATACACCTCACCGCCGCGCG-3′). The amplification product was digested with *Eco*RI and *Xba*I, and ligated into pPICZαA behind the α-factor signal sequence. The *P. pastoris* strain X-33 was transformed by electroporation using 10 μg of the *Pme*I-linearized construct, and transformants were selected on YPDS/Zeocin plates. A 3-ml preculture medium (BMGY, pH 6.0) was inoculated with freshly prepared single colonies and cultured for 2 d at 30 °C with vigorous shaking (200 rpm). The cells were collected by centrifugation, resuspended in 20 ml of inoculation medium (BMMY, pH 6.0), and incubated at 30°C with shaking at 200 rpm. Methanol (400 μl) was added to the culture medium every day. After 5 d of induction, the culture was centrifuged and the resulting supernatant medium was recovered and tested for enzyme activity. A 16-ml aliquot of the medium was concentrated to 260 μl, diluted with 12 ml of 20 mM citrate–phosphate buffer (pH 5.2), and concentrated by ultrafiltration on an Amicon Ultra-15 with a cut-off of 10 kDa (Millipore). The dilution/concentration process was repeated twice. After Biospin column 30 (Bio-Rad) treatment, protein concentration was measured using the Protein Assay reagent (Bio-Rad). SDS–PAGE was performed on a 10% polyacrylamide gel that was stained with Coomassie Brilliant Blue-R250.

Enzyme assay of the recombinant enzyme

Unless otherwise specified, recombinant enzyme preparations were incubated with substrates in 20 mM citrate–phosphate buffer (pH 5.2), 0.02% sodium azide, at 30 °C. For the determination of substrate specificity and effects of pH and temperature, enzyme amount and incubation time were adjusted to achieve linear production of fructose during the incubation period. The reaction was terminated by heating at 95 °C for 5 min. Timothy carbohydrate extracts were prepared from cold-acclimated timothy crown tissues sampled in the field in Sapporo during December 2007. One gram fresh weight (FW) of the tissues was boiled in 5 ml of distilled water. Levan for use as a substrate was prepared by 80% EtOH precipitation of products generated by the PpFT1 (timothy 6-SFT) recombinant enzyme with 1 M sucrose (Tamura *et al.*, 2009). Inulin, 1-kestotriose, 1,1-kestotriose, and 1,1,1-kestotetraose (Wako Pure Chemical Industries, Ltd) were used as substrates. The mean DP of levan and of inulin was estimated as the ratio of the amounts of fructose to glucose after hydrolysation with 0.06 M HCl. The enzymic products generated from timothy carbohydrate extract as a substrate were analysed by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD) (DX-500; Dionex) with a Carbo Pac PA-1 anion-exchange column (Dionex) as described in Tamura *et al.* (2009). Measurements of fructose and glucose were performed by HPLC using Shodex KS-802 and KS-803 columns (Showa Denko), and an L-2490 refractive index detector (Hitachi) as described by Yoshida *et al.* (1998). Hydrolase activities, including FEH and invertase activities, were evaluated by the amount of generated fructose.

Enzyme assay of crude enzyme extracts

Crude enzymic solutions were extracted from ~0.3 g of stubble or ~0.5 g of haplocorms using 20 mM citrate–phosphate buffer (pH 5.2) containing 1 mM dithiothreitol (DTT). Following centrifugation at 8000 *g* for 15 min, proteins in the resulting supernatants were concentrated by precipitation with 70% ammonium sulphate. The pellets were dissolved in 0.5 ml of 20 mM citrate–phosphate buffer (pH 5.2) with 1 mM DTT and desalted using a Biospin-30 column (Bio-Rad). Timothy levan, for use as a substrate, was extracted in boiling water from defoliated leaves that had been illuminated for 24 h in hydroponic culture. A 20-μl aliquot of the enzyme solution was incubated with 20 μl of 8.7 mM timothy levan in 20 mM citrate–phosphate buffer (pH 5.2) at 30 °C for 4 h. The reaction was terminated at 95 °C for 3 min. The amount of fructose released from fructans was measured by HPLC as described above. Total protein content in the enzyme solution was measured as described above.

Quantitative real-time RT-PCR

RNA extraction, cDNA preparation, and real-time RT-PCR were performed as described in Tamura *et al.* (2009). The primer pairs used in the real-time PCR for *Pp6-FEH1* were tyft81-DF (5′-CATTGGGTTAGCTAGCTCGT-3′) and tyft81-DR (5′-ACTACCCGACAGAACACTAG-3′), and for the ubiquitin gene (the internal reference gene) were ubi-RT-2F (5′-CAA-GAAGCGCAAGAAGAAG–3′) and ubi-RT-2R (5′-GTCGTC-GACCTTGTAGAACT-3′). The expression level of *Pp6-FEH1* was normalized against ubiquitin gene expression and adjusted against the expression levels of 0-h samples, which were deemed to be 1.0.

Measurement of carbohydrate content

Total water-soluble carbohydrates were extracted from ~0.3 g of stubble or ~0.5 g of young haplocorms by boiling the samples for 1 h in deionized water containing 1 mg ml^{-1} propylene glycol; the propylene glycol was added as an internal standard. Fructans in the extract were separated and measured by HPLC as described in Yoshida *et al.* (1998).

Statistical analysis

Student *t*-tests were performed on gene expression levels, enzyme activities, and fructan content using JMP 9 software (SAS).

Results

Cloning of a candidate FEH cDNA from timothy

A cDNA library derived from crown tissues was probed with a partial cDNA that had high similarity to vacuole invertases of Poaceae plants. In order to identify cDNAs from fructan-metabolism-related enzymes, in addition to the previously identified *PpFT1* coding FT of timothy (Tamura *et al.*, 2009), clones that were clearly positive for the probe used for *PpFT1* cloning were eliminated. This screening strategy identified several cDNAs with similarity to vacuolar invertase and FT and three cell wall invertase-like cDNAs, two of which only had partial sequences. One of the clones was a full-length cDNA, designated *Pp6-FEH1* (see below), that had 2139 bases and included an open reading frame of 1806 bases (AB583555). This clone was deduced to encode 601 amino acids containing three conserved amino acid motifs (WMS/NDPNG, RDP, and EC) that are suggested to be essential for β-fructosidase activity (Verhaest *et al.*, 2005) (Fig. 1). The amino acid corresponding to aspartic acid 239 in *Arabidopsis* cwINV1, which is important for binding and hydrolysis of sucrose (Le Roy *et al.*, 2007a), was substituted by methionine (278 from the first Met). An isoleucine (142) was located at the position corresponding to the serine 101 in *Cichorium intybus* 1-FEH IIa that is important for inhibitory binding of sucrose (Verhaest

et al., 2007; Le Roy *et al.*, 2008). The putative mature protein was predicted to have a pI of 5.0, molecular mass of 63.4 kDa, and have five *N*-glycosylation sites (N-X-S/T). The predicted amino acid sequence showed highest identity (69%) to wheat 6-FEH (Van Riet *et al.*, 2006). A phylogenetic comparison of the amino acid sequences of vacuolar invertases and FEHs from a range of species indicated that *Pp6-FEH1* was a member of a subgroup that includes wheat 6-FEH and several cell wall invertases in the Poaceae (Fig. 2).

Characterization of the Pp6-FEH1 recombinant protein

A partial cDNA containing the predicted mature protein region of *Pp6-FEH1* (Fig. 1) was ligated in-frame behind the α-signal sequence and heterologously expressed in *P. pastoris*. The recombinant Pp6-FEH1 showed the expected mass of 78 kDa on an SDS–PAGE gel (Fig. 3). HPAEC-PAD analysis of the timothy carbohydrate extract showed peaks that putatively corresponded to levan with sequential DPs (up to >50), in addition to low molecular weight sugars such as sucrose (Fig. 4; Supplementary Fig. S1, available at *JXB* online). The reaction mixture with the recombinant Pp6-FEH1 showed a decrease in the areas of the peaks corresponding to levan and an increase in that for fructose (Fig. 4; Supplementary Fig. S1 at *JXB* online). This indicated that the recombinant Pp6-FEH1 had 6-FEH activity. The decrease in peak area corresponding to sucrose after 4 h, suggesting that the recombinant Pp6-FEH1 also had invertase activity, was also confirmed (Fig. 4; Supplementary Fig. S1 at *JXB* online). The recombinant Pp6-FEH1 showed the temperature and pH dependence expected of FEH: optimal temperature was ~30 °C and optimal pH was 5.5 (Fig. 5). The activity of Pp6-FEH1 remained relatively high even at 4 °C, with up to 38% of its activity at 30 °C.

In order to characterize the enzymic properties of recombinant Pp6-FEH1 in detail, substrate specificities were investigated using a 1 mM concentration of different types of fructan (Table 1). Of the fructans tested, Pp6-FEH1 most efficiently hydrolysed levan (mean DP 20) with a relative activity four times greater than for 6-kestotriose (DP 3), which has the same linkage form as levan. Pp6-FEH1 also showed hydrolase activity for β(2,1)-linked fructans (DP 3 to mean 28), although the activity was lower than those for β(2,6)-linked fructans. Hydrolase activity for sucrose was quite low, but the occurrence of non-negligible activity towards 10 and 100 mM sucrose showed the intrinsic invertase activity of Pp6-FEH1. When a mixture of levan and sucrose is used as a substrate, an estimate of FEH activity is obtained by subtracting the amount of fructose released by invertase activity (approximated from the glucose production) from the total amount of fructose by hydrolase activity (see Table 1). Using this formula, the inhibitory effect of sucrose was evaluated as 4% at 10 mM sucrose and 23% at 100 mM sucrose.

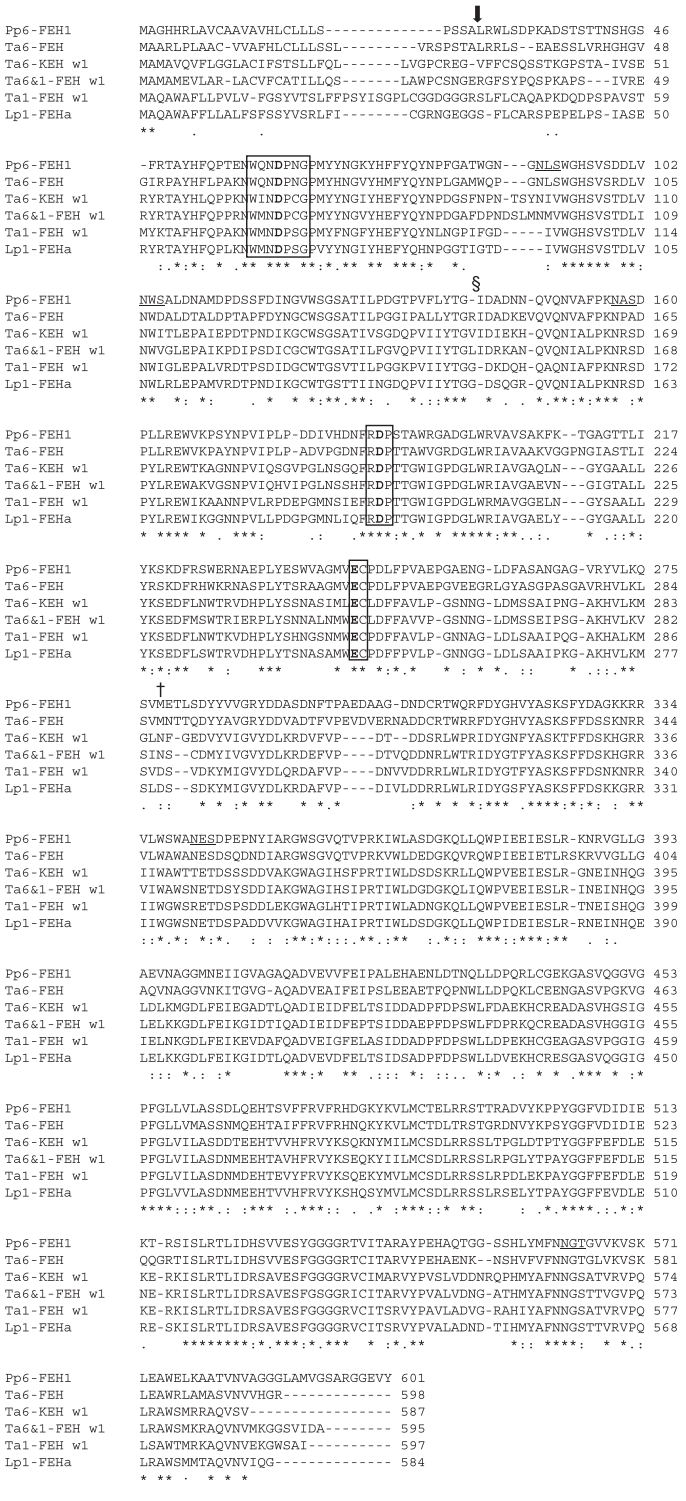

Fig. 1. Alignment of the amino acid sequences of Pp6-FEH1 and FEHs from Poaceae species. The three boxed regions indicate conserved motifs that are crucial for β-fructosidase catalysis. The three carboxylic acids implicated in catalysis are in bold. The amino acids homologous to Ser101 of chicory 1-FEH IIa are indicated by §. † shows the amino acids homologous to Asp239 of AtcwINV1. The putative glycosylation sites of Pp6-FEH1 are underlined. The arrow indicates the predicted N-terminus of the mature Pp6-FEH1 protein.

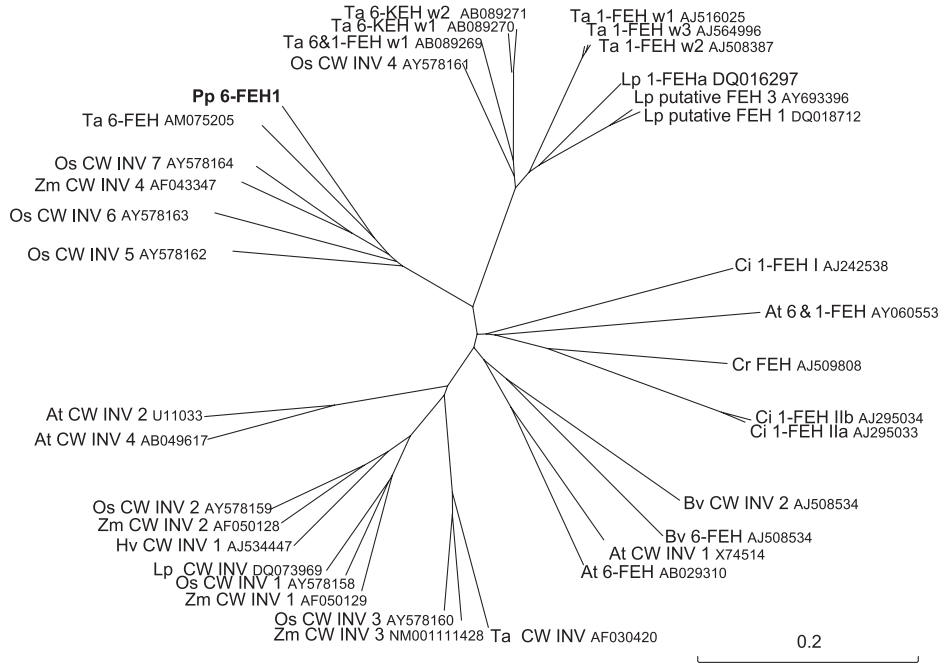

Fig. 2. Phylogenetic tree of FEHs and cell wall invertases (CW INV) of plants based on predicted amino acid sequences. Scale bar indicates branch length. GenBank accession numbers of the genes used in this analysis are given after the gene and species abbreviation. Abbreviations for the species are: At, *A. thaliana*; Bv, *B. vulgaris*; Ci, *C. intybus*; Cr, *Campanula rapunculoides*; Hv, *Hordeum vulgare*; Lp, *L. perenne*; Os, *Oryza sativa*; Pp, *P. pratense*; Ta, *Triticum aestivum*; Zm, *Zea mays*.

Analysis of Pp6-FEH1 *transcripts in stubble and haplocorms after defoliation*

In seedling stubble, the level of expression of *Pp6-FEH1* rapidly increased (~7-fold) within 3 h of defoliation; expression then decreased but was nevertheless maintained at a significantly higher level than in non-defoliated tissues up to 72 h after defoliation (Fig. 6A). In the control (non-defoliated seedlings), the *Pp6-FEH1* transcript level increased at 3 h but was significantly lower than in the defoliated stubble, and was then maintained at the same level as at 0 h (Fig. 6A). The increase in 6-FEH activity began within 3 h of defoliation, and at 24 h the activity was ~4-fold greater than at 0 h. In the control, 6-FEH activity remained at the same level as at 0 h throughout the tested period (Fig. 6B). Fructan content was significantly lower than that in control after 24 h (Fig. 6C). The mean lengths of newly elongated leaves after defoliation were 9.1 mm at 8 h and 22.0 mm at 24 h.

Next, changes in *Pp6-FEH1* expression in young haplocorms after defoliation of timothy plants were investigated. An increase in *Pp6-FEH1* expression was evident within 3 h of defoliation at ~7-fold of the control level; the amount of transcripts had rapidly increased by 7 h after defoliation (Fig. 7A; Supplementary Fig. S2A at *JXB* online). At 4 d after defoliation, *Pp6-FEH1* transcripts were ~140-fold more abundant than those at 0 h (Fig. 7A; Supplementary Fig. S2A at *JXB* online). Thereafter, the transcript level decreased but, even at 14 d, was >15-fold higher than at 0 h. However, the change in *Pp6-FEH1* transcript levels in control plants was much smaller than in defoliated plants (Fig. 7A; Supplementary Fig. S2A at *JXB* online). An increase in 6-FEH activity at 7 h after defoliation was present compared with the control; the significantly higher level of activity was maintained for 7 d (Fig. 7B; Supplementary Fig. S2B at *JXB* online). The amounts of fructans in defoliated haplocorms were significantly lower than those in the control after 4 d and were about one-ninth the abundance of those in control plants at 14 d (14 mg g^{-1} FW compared with 130 mg g^{-1} FW; Fig. 7C; Supplementary Fig. S2C at *JXB* online). The mean length of newly elongating leaves after defoliation was 9.7 mm at 7 h and 95.2 mm at 4 d.

Discussion

Pp6-FEH1 *encodes a 6-FEH for highly polymerized levan*

Phylogenetic analysis of amino acid sequences indicated that Pp6-FEH1 showed highest similarity to wheat 6-FEH (Van Riet *et al.*, 2006) and was classified with other Poaceae cell wall invertases (Fig. 2). Pp6-FEH1 does not have the Asp239 homologue of AtcwINV1, which has been proposed as a reliable determinant for discriminating true invertases from defective invertases/FEHs (Le Roy *et al.*, 2007a). The recombinant Pp6-FEH1 showed a higher substrate specificity for β(2,6)-linked fructans than for β(2,1)-linked ones; in particular, its activity for levan was 10-fold more than for inulin. This attribute indicates that Pp6-FEH1 can be classified as a 6-FEH. The preference of Pp6-FEH1 for

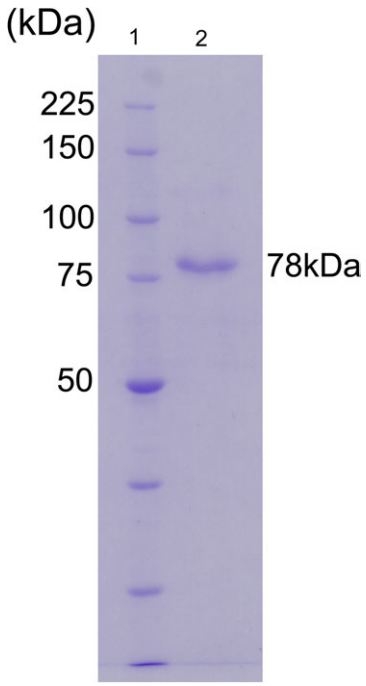

Fig. 3. SDS–PAGE of the concentrated supernatant from the culture of transformed *P. pastoris* expressing a recombinant Pp6-FEH1. Lane 1, molecular mass marker proteins with their masses indicated at the left; lane 2, recombinant Pp6-FEH1 (1.14 μg of protein) with predicted mass 78 kDa.

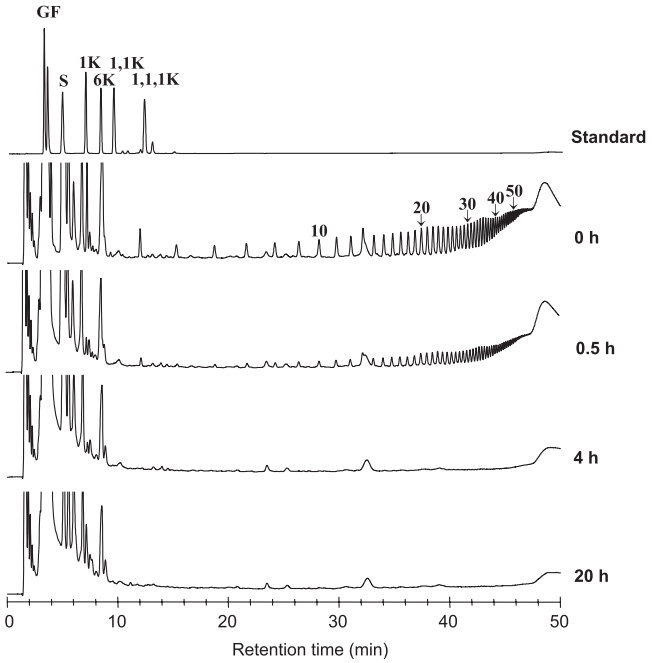

Fig. 4. Anion exchange HPLC analysis of products generated by recombinant Pp6-FEH1 in the presence of timothy carbohydrate extracts. A 180-μl aliquot of the extract was incubated with 20 μl of the recombinant enzyme (57 μg) for 0, 0.5, 4, and 20 h. Abbreviations for each sugar peak in standard are: G, glucose; F, fructose; S, sucrose; 1K, 1-kestotriose; 6K, 6-kestotriose; 1,1K, 1,1-kestotetraose; 1,1,1K; 1,1,1-kestopentaose. The numbers indicate the putative DPs of β(2,6)-linked linear fructans.

β(2,6)-linked fructans as substrate is consistent with the fact that timothy mainly accumulates β(2,6)-linked fructans. Indeed, the recombinant Pp6-FEH1 completely degraded the native fructans in timothy (Fig. 4).

In wheat, four genes, *6-KEH w1* and *w2* (Van den Ende *et al.*, 2005), *6&1-FEH* (Kawakami *et al.*, 2005), and *6-FEH* (Van Riet *et al.*, 2006) have been reported to encode FEHs that show considerable activity for degradation of β(2,6)-linked fructosyl residues. However, Pp6-FEH1 showed a different substrate specificity from these wheat FEHs. Pp6-FEH1 had a clearly higher activity for β(2,6)-linked fructans with high DPs than for those with a low DP (levan with mean DP 20 compared with 6-kestotriose with DP 3, Table 1). By contrast, wheat 6-FEH shows almost similar substrate specificities for β(2,6)-linked fructans with different DPs such as 6-kestotriose, phlein (DP 4–12), and levan (DP ±72) (Van Riet *et al.*, 2006). The remarkable difference in substrate specificity for β(2,6)-linked fructans with different DPs is also shown by wheat 6-KEHs and 6&1-FEH (Kawakami *et al.*, 2005; Van den Ende *et al.*, 2005), which have a preference for certain small oligomers as substrates. The high affinity of Pp6-FEH1 for β(2,6)-linked fructans with high DPs might be related to the fact that timothy accumulates mainly levan with high DPs (up to 90), in contrast to other temperate grasses (Suzuki, 1968; Cairns *et al.*, 1999). In 1-FEH, it has been reported that the presence or absence of a glycosyl chain in the cleft between the N- and C-terminal domains is important for the binding of high-DP inulins; introduction of a glycosyl chain into *C. intybus* 1-FEH IIa prevents inulin binding and degradation (Le Roy *et al.*, 2007*b*). However, 6-FEHs seem to bind to levan in a different way and at a different location (Le Roy *et al.*, 2007*b*). Indeed, the predicted mass of *Pichia* recombinant Pp6-FEH1 from SDS–PAGE suggested the addition of glycosyl chains (Fig. 3). Pp6-FEH1 has a glycosylation site in the cleft region (Fig. 1, 341–343 from first Met) like other 6-FEHs and cell wall invertases (Le Roy *et al.*, 2007*b*).

Purified native enzyme and the *Pichia* recombinant wheat 6-FEH showed extremely low activity against β(2,1)-linked fructans [at least lower than 1/100 compared with bacterial levan (DP ±72) and phlein (DP 4–12) (Van Riet *et al.*, 2006)]. However, the *Pichia* recombinant Pp6-FEH1 had significant activity against β(2,1)-linked fructans of 6–18% of that against levan. This indicates that Pp6-FEH1 has weak 1-FEH activity in addition to its 6-FEH activity. Wheat 6&1-FEH has both 6-FEH and 1-FEH activity with a substrate preference for bifurcose (1&6-kestotetraose), although it shows little or no activity for high-DP fructans (Kawakami *et al.*, 2005), and is therefore different from Pp6-FEH1.

In contrast to wheat 6-FEH, which is not inhibited by sucrose (Van Riet *et al.*, 2006), a small decrease in 6-FEH activity was found when Pp6-FEH1 was incubated with

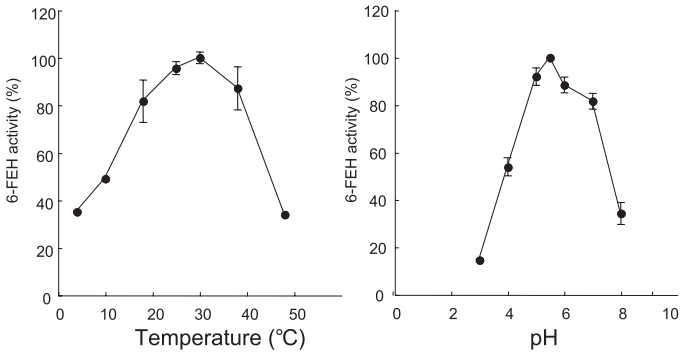

Fig. 5. Effect of temperature and pH on the 6-FEH activity of recombinant Pp6-FEH1. Activity is expressed as a percentage of the maximum activity calculated by the amount of fructose released after incubation of 0.6 μg of recombinant Pp6-FEH1 with 1 mM levan with mean DP 20.

Table 1. Substrate specificity of recombinant Pp6-FEH1 expressed in *P. pastoris*

Activity is shown as the value relative to activity with 1 mM levan as substrate. The value of 100% corresponds to 11.6 μmol mg^{-1} protein min^{-1}.

Substrate	Linkage form	DP	Relative activity (%)
1 mM 6-kestotriose	β(2,6)	3	25
1 mM levan	β(2,6)	20[a]	100
1 mM 1-kestotriose	β(2,1)	3	7
1 mM 1,1-kestotetraose	β(2,1)	4	18
1 mM 1,1,1-kestopentaose	β(2,1)	5	18
1 mM inulin	β(2,1)	28[a]	6
1 mM sucrose		2	<<1
10 mM sucrose		2	2
100 mM sucrose		2	15
Mixture of 1 mM levan+10 mM sucrose			97
Mixture of 1 mM levan+100 mM sucrose			87

[a] Mean DP.

a combination of 10 mM or 100 mM sucrose and 1 mM levan (Table 1), although the degree of inhibition was very low compared with those shown by 1-FEHs (Van den Ende *et al.*, 2003*a*; Lothier *et al.*, 2007; Verhaest *et al.*, 2007; Asega *et al.*, 2008). Like other FEHs, Pp6-FEH1 shows extremely low substrate specificity to sucrose compared with levan at the same molar concentration, but has considerable invertase activity against high concentrations of sucrose (Table 1). Indeed, the recombinant Pp6-FEH1 almost completely degraded the native fructans of timothy into glucose and fructose after a long incubation (Supplementary Fig. S1 at *JXB* online). Verhaest *et al.* (2007) proposed a relationship between sucrose inhibition and the single amino acid corresponding to Ser101 in *C. intybus* 1-FEH IIa. This proposal was based on the results of three-dimensional structural analysis and of site-directed mutagenesis. FEHs with a small amino acid, such as glycine or serine, at the site corresponding to Ser101 were strongly inhibited by sucrose, whereas those with a hydrophobic amino acid, such as valine, leucine or isoleucine (as in Pp6-FEH1), were weakly inhibited by sucrose (Verhaest *et al.*, 2007). Similarly to Pp6-FEH1, wheat 6-KEH w1 and w2 with a valine positioned at Ser101 showed weak (40–60%) inhibition by 100 mM sucrose against 4 mM 6-kestotriose as a substrate (Van den Ende *et al.*, 2005). Weak invertase activity and sucrose inhibition might be the consequence of 'an unstable substrate configuration' (Le Roy *et al.*, 2008) in Pp6-FEH1. However, the arginine residue in wheat 6-FEH at the site corresponding to Ser101 seemed to prevent binding of sucrose in both the inhibitor and substrate configuration (Verhaest *et al.*, 2007).

Comparisons of substrate specificities of 6-FEHs and related enzymes suggest that Pp6-FEH1 is a novel type of 6-FEH, although a definitive evaluation still awaits comparison using the same substrates and under the same conditions. The different enzymic properties of wheat 6-FEH and Pp6-FEH1, despite their high similarity in amino acid sequence, support the prediction that introduction of a limited number of mutations in the common ancestor might have diversified the function of FEHs. There are also genes encoding enzymes with unexpected 6-FEH activity in non-fructan plants such as beet and *Arabidopsis* (Van den Ende *et al.*, 2003*b*; De Coninck *et al.*, 2005). For example, AtcwINV6 of *A. thaliana* shows a somewhat higher affinity for high-DP levan and phlein than for 6-kestotriose. As mentioned above, the relationship between DP specificity and the structure of 6-FEH enzymes remains uncertain. In addition to comparison of amino acid sequences of 6-FEH from fructan and non-fructan plants, approaches using chimeras and directed point-mutations in recombinant enzymes might elucidate the motifs or amino acids responsible for the DP preference of 6-FEHs.

Pp6-FEH1 *plays a role in fructan mobilization after defoliation*

It was demonstrated that transcription of *Pp6-FEH1* was induced within 3 h of defoliation, and was followed by an increase in 6-FEH activity in both seedling stubble and young haplocorms (Figs 6, 7; Supplementary S2 at *JXB* online). With regard to the enzymic function of Pp6-FEH1, these findings suggest that induction of *Pp6-FEH1* expression plays a role in the initiation of fructan degradation and mobilization after defoliation in timothy. They also support the report of Yamamoto and Mino (1989) of *de novo* synthesis of FEH enzyme after defoliation. The present study provides the first indication of defoliation-induced transcription of FEH in grasses. The chicory root shows induction of 1-FEH II transcription within 1 d of defoliation (Van den Ende *et al.*, 2001), while induction of 1-FEH transcripts occurs in the rhizophore of *Vernonia herbacea* 20 d after defoliation (Asega *et al.*, 2008). This induction response is similar to that found here for *Pp6-FEH1* in timothy stubble.

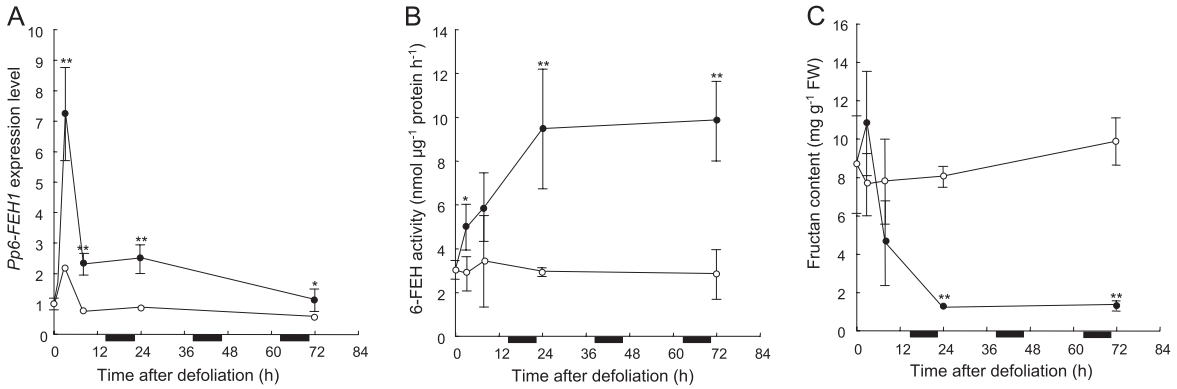

Fig. 6. *Pp6-FEH1* expression levels, 6-FEH activity, and fructan content in stubble of timothy seedlings after defoliation. Black bars indicate dark conditions. In (A), (B), and (C), the closed symbols indicate values after defoliation and the open symbols indicate values in the control. Expression levels of *Pp6-FEH1* normalized against those of the ubiquitin gene, were measured by quantitative real-time RT-PCR (A). Activity of 6-FEH in crude enzyme extracts was measured as amount of fructose released from levan (B). The amount of fructan was measured by HPLC (C). Results are given as the mean±SD (except where the SD was smaller than the size of the symbol); n=3. Significant differences between defoliated and control plants are indicated by *(P<0.05) or **(P<0.01).

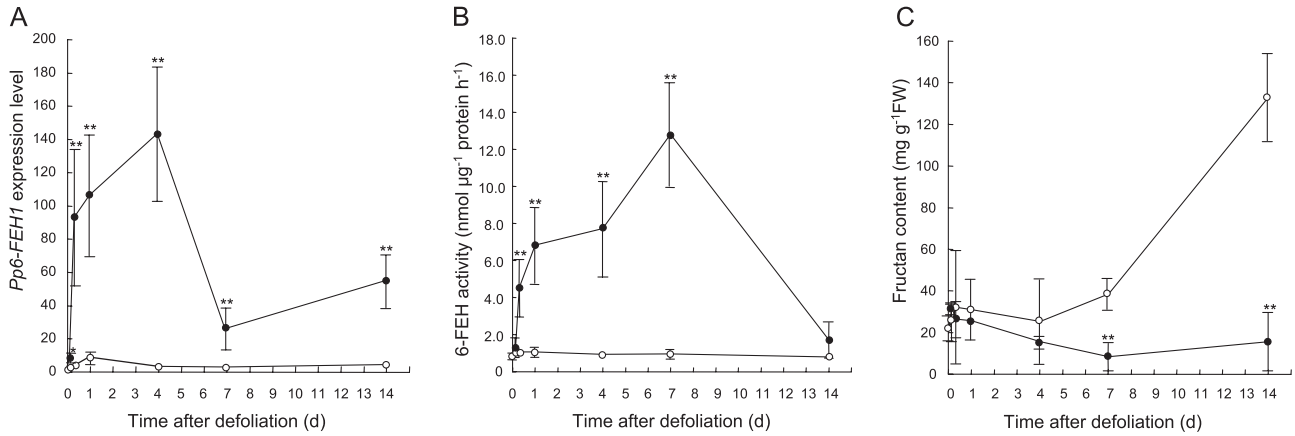

Fig. 7. *Pp6-FEH1* expression levels, 6-FEH activity and fructan content in haplocorms of timothy after defoliation. In (A), (B), and (C), the closed symbols indicate values after defoliation and the open symbols indicate values in the control. The level of expression of *Pp6-FEH1* was determined by quantitative real-time RT-PCR and normalization against the ubiquitin gene (A). Generation of fructose by crude enzyme extracts incubated with levan was measured as 6-FEH activity (B). The amount of fructan was measured by HPLC (C). Results are given as the mean±SD (except where the SD was smaller than the size of the symbol); n=3. Significant differences between defoliated and control plants are indicated by *(P<0.05) or **(P<0.01).

The maximum transcript level of *Pp6-FEH1* after defoliation, compared with 0 h, was much higher in haplocorms than in seedling stubble: ~140-fold increase in haplocorms at 4 d compared with 7-fold in stubble at 3 h after defoliation). However, the level of induction of FEH activity showed less difference: 15-fold at 7 d in haplocorms compared with 4-fold at 24 h in seedling stubble. Post-transcriptional regulation of Pp6-FEH1 or other FEH enzymes might be involved in the different relationship between *Pp6-FEH1* transcript level and 6-FEH activity in the two tissues. The induction of *Pp6-FEH1* transcripts in haplocorms was maintained for 4 d after defoliation, whereas in the stubble of seedlings the transcript level dropped rapidly by 8 h. The rate of decrease of fructan content in the haplocorms and seedling stubble appeared to correlate with the fluctuations in levels of *Pp6-FEH1* transcripts (Figs 6, 7).

What factors might affect the induction of *Pp6-FEH1* expression? Exogenous application of sucrose, fructose, or glucose to excised pseudostems of orchardgrass appears to suppress any increase in FEH activity (Yamamoto and Mino, 1987). In the present study, these sugars rapidly decreased in the stubble and haplocorms of timothy after defoliation (data not shown). The decrease in photosynthetic assimilates after defoliation might induce an increase in *Pp6-FEH1* transcripts in order to mobilize carbon sources. Morvan *et al.* (1997) reported that FEH activity in *L. perenne* was inhibited by uniconazole, an inhibitor of the biosynthesis of gibberellin, and also

showed an increase in activity following treatment with gibberellic acid. However, an increase in gibberellin content in stubble after defoliation has not been confirmed (Morvan-Bertrand *et al.*, 2001). In orchardgrass, exogenous gibberellic acid, cytokinin, or 8-bromo-cAMP all increased FEH activity in pseudostems (Yamamoto and Mino, 1998). A future study of the transcriptional analysis of *Pp6-FEH1*, including identification of inducing factors and *cis*-acting elements in the promoter, should help to elucidate the mechanism of transcriptional regulation of *FEH* genes after defoliation.

The regulation of latent FEH enzyme activity by means of sucrose inhibition could not be completely excluded in timothy, but at least for the Pp6-FEH1 enzyme the effect of such inhibition would be relatively small compared with that of induction of transcripts. In *L. perenne*, a grass species frequently used for grazing due to its high regrowth performance, activity of both 6-FEH and 1-FEH is strongly inhibited by sucrose (Marx *et al.*, 1997; Lothier *et al.*, 2007). The major advantage of sucrose inhibition of FEH activity is a rapid shift from net fructan biosynthesis to net fructan degradation when sucrose is lacking (Verhaest *et al.*, 2007). It will be of interest to determine whether the difference in the regulation mechanism of FEH activity in forage grass species affects the speed of regrowth after defoliation.

Subcellular localization of Pp6-FEH1

As fructans are mainly accumulated in the vacuole, it is generally believed that fructan-metabolizing enzymes must be present there (Wanger and Wiemken, 1986). Two factors suggest localization of Pp6-FEH1 in the vacuole: a low pI (5.0), which is characteristic of vacuolar enzymes; and a weak, but intrinsic, response to inhibition by sucrose, which is present at a high concentration in the vacuole. However, the possible localization of Pp6-FEH1 to the apoplast cannot be excluded as FEH activity in the apoplast has been reported by several studies (Livingston and Hensen, 1998; Van den Ende *et al.*, 2005).

Supplementary data

Supplementary data can be found at *JXB* online.

Fig. S1. HPAEC-PAD chromatograms corresponding to those in Fig. 4 but redrawn using an extended *y* axis scale.

Fig. S2. Bar graph representation of the line graphs in Fig. 7.

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